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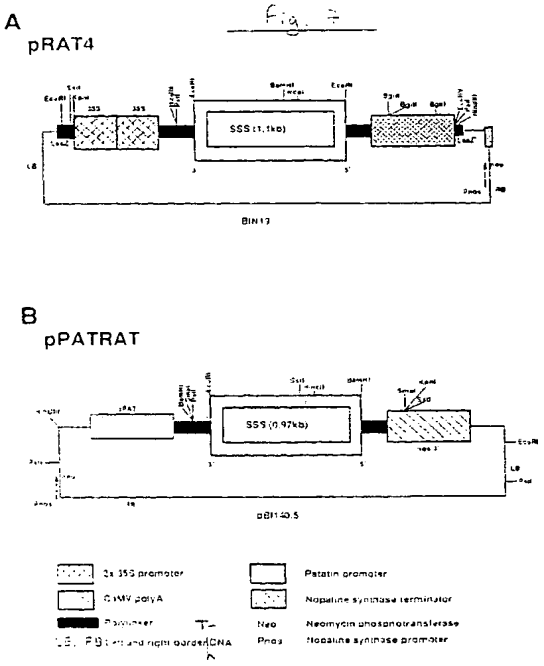
(54) Improvements in or relating to soluble starch synthase

(57) Disclosed is altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants, together with a nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or antisense orientation to a promoter operable in a plant, and host cells and plants comprising the sequence.



EP 0 779 363 A2

in the name of Institut Für Genbiologische Forschung Berlin GMBH. The PCT application includes the European Patent Office in the list of designated territories.

WO 96/15248 discloses the nucleotide sequence of a full length cDNA clone ("SSSA") said to encode an isoform of soluble starch synthase enzyme from potato, together with the predicted amino acid sequence of the enzyme. The application further discloses the use of a 1.2kb portion of the cDNA clone, operably linked in the antisense orientation to the CaMV 35S promoter, to transform potato plants. In addition WO 96/15248 discloses the sequence of a cDNA clone ("SSSB") said to encode a second isoform of the potato soluble starch synthase. Similarly, a portion (1.8kb) of this sequence was introduced into potato plants in the antisense orientation.

In fact, the present inventors have found that the nucleotide sequence disclosed in WO 96/15248 contains an error, causing a frame shift, such that most of the predicted amino acid sequence is incorrect.

It was found that the transformed plants disclosed in WO 96/15248 had reduced enzyme activity. Starch obtained from the tubers of the transformed plants was found to have altered properties compared to starch obtained from control wild type plants. It was stated that the starch from the transformed plants exhibited a lower viscosity onset temperature than starch from control plants. (By way of explanation, when aqueous suspensions of starch granules are heated, the granules swell and absorb water, in a process known as gelatinisation. A number of techniques are available for the analysis of gelatinisation, a particularly convenient method being differential scanning calorimetry or the viscoamylograph, in which the viscosity of a stirred starch suspension is monitored under a defined temperature/time regime. Such analysis typically shows a particular temperature, the "viscosity onset temperature", at which the process of gelatinisation begins and which causes a marked increase in viscosity of the starch suspension).

In a few instances, the transformed plants disclosed in WO 96/15248 gave rise to starch in which the "Verkleisterungstemperatur" (equivalent to the viscosity onset temperature, V) was 2 to 3°C lower compared to starch from equivalent, but untransformed, plants. However, it is apparent that the results of subsequent experiments (described in example 13 in the document) gave a value of V for starch from control plants which was lower than that found for starch from transformed plants in previous experiments. Accordingly, the person skilled in the art is not able to deduce that starch from the transformed plants described in WO 96/15248 displayed a viscosity onset temperature which was consistently significantly lower than that of control plants.

Summary of the Invention

In a first aspect the invention provides a polypeptide obtainable from a soluble extract of potato tubers and having starch synthase activity, in substantially isolated form.

Typically the polypeptide will have an apparent molecular weight, as judged by SDS-PAGE, in the range 100-140 kDa, or will be a functional equivalent of such a polypeptide. More particularly, the polypeptide may have an apparent molecular weight of 140, 120 or 110 kDa. Particular functional equivalents envisaged are breakdown products of the polypeptide, which seem to occur naturally. Another particular functional equivalent is the polypeptide obtainable from developing tubers of the Desiree cultivar, which polypeptide has an apparent molecular weight, as judged by SDS-PAGE, of 100 kDa. Typically the polypeptide will comprise the amino acid sequence shown in Figure 6.

In another aspect the invention provides a nucleic acid sequence directing the expression of at least a portion of one of the polypeptides defined above. Preferably the sequence comprises at least 200-300bp, more preferably at least 300-600bp, and most preferably in excess of 600bp. Typically the nucleic acid sequence will comprise the nucleotide sequence shown in Figure 6, although those skilled in the art will appreciate that, due to the degeneracy of the genetic code, a nucleotide sequence substantially different to that shown in Figure 6 may encode a polypeptide having substantially the same amino acid sequence as that shown in Figure 6. Such nucleic acid sequences are to be considered as functional equivalents and thus fall within the scope of the present invention. Other functional equivalents are those nucleic acid sequences which are not substantially different and which may hybridise, under standard laboratory hybridisation conditions, to either strand of the nucleotide sequence shown in Figure 6.

Comparison with known starch synthase sequences, with the benefit of the disclosure herein, will enable those skilled in the art to identify regions of the sequence shown in Figure 6 which are not evolutionarily conserved, and so more amenable to alteration (e.g. addition, deletion or substitutions), whilst retaining functional equivalence.

Desirably such functional equivalents will possess at least 80% sequence identity, preferably at least 85% sequence identity, and more preferably at least 90% sequence identity with the nucleotide sequence shown in Figure 6. Desirably the nucleotide sequence of the invention, or a functional equivalent sequence will, when introduced into a suitable plant in a suitable manner (known to those skilled in the art), alter the synthesis of starch in the plant.

For the purposes of the present specification, the sequences encoding polypeptides with starch synthase activity, or portions of such sequences, disclosed in WO 96/15248 are not considered as functional equivalents of the sequence shown in Figure 6.

In a particular embodiment, the invention provides a nucleic acid sequence comprising at least 200 bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably

undergoing such modifications as can alter the pasting properties thereof.

"Equivalent, non-transformed" plants are those plants which have substantially identical genotypes to the plants of the invention, with the exception of the introduced nucleic acid sequence present in the transformed plants of the invention.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which;

Figure 1 shows the elution profile of starch synthase from developing Desiree potato tubers on a first Mono Q™ anion-exchange column. Partially purified starch synthase, after DEAE-Sepharose and Blue Sepharose chromatography, was applied to a 1ml Mono Q™ column at pH 7.5. The enzyme was eluted with a 25ml gradient of 0-450 mM KCl at 0.5 ml.min^{-1}. Samples (20 µl) of each 1 ml fraction were assayed for starch synthase activity (-●-), and absorbance at 280 nm (-);

Figure 2 shows the activity and protein in fractions of purified starch synthase from a second Mono Q™ column of peak I and peak II. Top panels show SDS-PAGE of fractions containing starch synthase activity. Each track contains 10 µl of fraction. Bottom panels show starch synthase activity in 20 µl samples from each 0.5 ml fraction;

Figure 3 shows the cross-reaction of antiserum to SSS to the purified starch synthases from mature Estima tubers and to extracts, soluble and granule-bound, from mature Estima and developing Desiree tubers. Samples (10 µl of purified soluble starch synthase, 20 µl of partially purified soluble starch synthase, 20 µl soluble extract and 20 µl of supernatant from granule-bound proteins) were subjected to SDS-PAGE and blotted, and then the blots were probed with antiserum to SSS, 1/2500 dilution. (1) purified preparation of starch synthase proteins from mature Estima. (2) Partially purified soluble starch synthase from mature Estima tubers. (3) Starch-granule-bound proteins from mature Estima tubers. (4) Soluble extract from developing Desiree tubers. (5) Starch-granule-bound proteins from developing Desiree tubers. Sizes of proteins were estimated from molecular weight standards on the same gels, and are indicated in kDa;

Figure 4 shows the immunoprecipitation of starch synthase activity in soluble extract from developing Desiree tubers with antiserum to SSS. Soluble extract was incubated with increasing volumes of pre-immune serum (○) and antiserum (◐), as described in Materials and Methods (below). After centrifugation the supernatant was assayed for starch synthase activity. Starch synthase activity is expressed as a percentage of activity of incubations containing 20 g.L^{-1} BSA in PBS. Values are from two separate experiments with the line joining the means;

Figure 5 shows native polyacrylamide gel electrophoresis of soluble extract from developing Desiree tubers stained for starch synthase activity. Soluble extract was incubated (as described in Materials and Methods) in the presence of (1) 20 g.L^{-1} BSA in PBS; (2) pre-immune serum, 1/1000 dilution; (3) antiserum to SSS, 1/1000 dilution; and (4) antiserum to the GBSS II from pea embryo. After centrifugation, the supernatant was mixed 5:1 with 2 g.L^{-1} bromophenol blue in 500 ml.L^{-1} glycerol and 40 µl was loaded onto the gel. The bands of starch synthase activity are indicated by arrows;

Figure 6 shows the DNA sequence of a cDNA clone for potato soluble starch synthase. The amino acid sequence of the encoded polypeptide is shown below in the single letter code. The ADP-glucose binding domain is boxed and the sequences identified by protein sequencing are underlined; and

Figure 7 shows a schematic representation of A) plasmid pRAT4 and B) plasmid pPATRAT.

EXAMPLES

Example 1

In this example are presented data on the identification and purification to homogeneity of the major isoform of soluble starch synthase from potato tuber.

MATERIALS AND METHODS

Plant material.

Potato tubers (*Solanum tuberosum* L.) of cultivars Desiree (developing) or Estima (mature) were used. Desirée

The dialysed eluate was applied to a first 1ml Mono Q™ column, equilibrated with medium B, as described above, except that all the fractions containing starch synthase activity were pooled together.

The Mono Q™ eluate was applied to a CHA-Sepharose column as described above, except that the column was 1.0 cm i.d., 20 cm long. The column was washed with 50 ml medium B containing 0.5 M sodium citrate and eluted with 80 ml medium B without citrate. The fractions with starch synthase activity were pooled and dialysed ovemight against 5 L of medium C.

The dialysed extract was applied to a second 1ml Mono Q™ column equilibrated with medium C, as described above. Fractions containing starch synthase activity were stored at -20°C.

Preparation of antibody.

The fractions containing starch synthase activity from 5 large-scale purifications were run on preparative sodium dodecyl sulphate (SDS)-polyacrylamide gels (as described below). The gel slices containing starch synthase proteins were electroeluted and the proteins dialysed against water, then freeze-dried. Protein (50 µg) was re-dissolved in 250 µl of phosphate-buffered saline (PBS), mixed with 250 µl Freund's complete adjuvant, and injected intramuscularly into a rat. Subsequent injections were of 75 µg protein dissolved in 250 µl PBS mixed with 250 µl Freund's incomplete adjuvant and were repeated at 14-day intervals. Serum was collected from 14 days after the third injection.

Assay of soluble starch synthase activity.

Soluble starch synthase activity was measured using the resin method as described in Jenner *et al.* (1994).

Preparation of crude soluble potato tuber extract.

Samples (0.5-2.0 g fresh weight) from either developing Desiree or mature Estima potato tuber were homogenised in 4 volumes of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 $g.L^{-1}$ sodium metabisulphite, 0.5 $mg.L^{-1}$ leupeptin, 0.7 $mg.L^{-1}$ pepstatin A and 50 $ml.L^{-1}$ glycerol, then centrifuged at 10,000g for 10 min. The supernatant is referred to as "soluble extract".

Partial purification of soluble starch synthase activity.

Crude soluble potato extract from mature Estima tubers (5-10 g fresh weight) was dialysed twice, each time against 1 L of buffer B for 1 hr. The dialysed extract was applied to a lml Mono Q™ column, equilibrated with medium B, as described above and the peak fraction of starch synthase activity (referred to as "partially purified soluble starch synthase") was stored at -20°C.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Protein samples were dialysed against distilled water then mixed 1:1 with double-strength sample buffer (Laemmli, U.K. (1970). Nature 227, 680-685) and boiled for 2 min immediately prior to application to gels. For granule-bound proteins, starch granules were washed twice in 20 $g.L^{-1}$ SDS at room temperature, boiled for 3 min at 100 $mg.ml^{-1}$ in sample buffer (Laemmli, U.K. (1970). Nature 227, 680-685) and then centrifuged at 10,000g for 10 min. The supernatant was applied to the gel.

Gels (10.2 cm long, 7.3 cm wide, 0.75 mm thick) were 75 $g.L^{-1}$ acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) and 1 $g.L^{-1}$ SDS and were run according to Laemmli (1970). Immunoblots were prepared and developed according to Bhattacharyya *et al.*, (1990) Cell 60, 115-122. The nitrocellulose filters were either incubated with crude rat serum followed by alkaline phosphatase-congugated goat anti-rat antiserum (Sigma, Poole, Dorset, UK) or the immunoglobulin fraction of rabbit serum to GBSS II from pea embryo (Smith (1990) Planta 182, 599-604), followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma).

Native polyacrylamide gel electrophoresis.

Gels (dimension as above, except lmm thick) of 90 $g.L^{-1}$ acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) were cast in 400 mM Tris-HCl (pH 8.6), 100 $ml.L^{-1}$ glycerol, 8 $g.L^{-1}$ glycogen and polymerised with 0.4 $g.L^{-1}$ ammonium persulphate and 0.2 $ml.L^{-1}$ N,N,N',N'-tetramethylethylenediamine (TEMED) and were overlaid with a stacking gel of 53 $g.L^{-1}$ acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) cast in 155 mM Tris-HCl (pH 6.8), 98 $ml.L^{-1}$ glycerol, polymerised with 0.5 $g.L^{-1}$ ammonium persulphate and 0.2 $ml.L^{-1}$ TEMED. Soluble extracts were mixed 5:1 with 2 $g.L^{-1}$ bromophenol blue in 500 $ml.L^{-1}$ glycerol immediately prior to loading. Gels were run at 4°C, at 175 mV in 190 mM

TABLE 1 (continued)

FRACTION	TOTAL ACTIVITY (μmol glucose incorporated min^{-1})	ACTIVITY RECOVERED (%)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (μmol glucose incorporated min^{-1}. mg $protein^{-1}$)
Peak I				
Mono Q (pH 8.0)	0.15	0.5	0.03	5.13
Peak II				
Mono Q (pH 7.5)	2.45	8.8	2.90	0.85
Cyclohexa-amylose	2.27	8.1	0.40	5.67
Mono Q (pH 8.0)	0.26	0.9	0.03	0.84

TABLE 2

INCUBATION	INHIBITION OF STARCH SYNTHASE ACTIVITY (%)
Pre-immune serum	0.3 ± 0.9
Antiserum to potato SSS	74 ± 4
Antiserum to pea GBSS II	9 ± 4
Antiserum to potato SSS + pea GBSS II	80 ± 8

Table 2 shows the immunoprecipitation of starch synthase activity in soluble extract from developing Desiree tubers. Soluble extract was incubated in the presence of antiserum (1/10 dilution of rat antiserum; or 1/5 dilution of the immunoglobulin fraction of rabbit serum to GBSS II from pea embryo; or 1/10 dilution of rat antiserum plus 1/5 dilution of the immunoglobulin fraction of rabbit serum to GBSS II), as described in Material and Methods. After centrifugation the supernatant was assayed for starch synthase activity. Values are percentage inhibition relative to controls in which BSA at 20 $g.l^{-1}$ in PBS was substituted for serum. The values are the mean of four experiments ± standard error.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions from the second Mono Q™ column for peak I showed that the distribution of a protein of 120 kDa matched the distribution of the starch synthase activity (Figure 2). Further chromatography on Mono Q™ did not eliminate contaminating proteins. SDS-PAGE of the fractions from the final Mono Q™ column for peak II showed that the distribution of the major protein of 110 kDa matched the starch synthase activity (Figure 2).

Antibodies raised to the 59 kDa starch-granule-bound protein (the GBSS I isoform) from pea embryo did not recognise any proteins from either peak I or peak II. Antibodies raised to the 77 kD GBSS II from pea embryo very weakly recognised the 120 and 110 kDa proteins from peak I and II respectively (data not shown).

Preparation of antibody.

In order to obtain sufficient protein for preparation of an antibody, peaks I and II from mature Estima tubers were combined and purified together in large-scale preparations (referred to as "soluble starch synthase", SSS). Both the 120- and 110 kDa proteins were excised and eluted from gels of the purified preparations and were injected into the same rat.

The antiserum to the SSS was used to probe blots of extracts from mature Estima and developing Desiree tubers (Figure 3). On all of the immunoblots, the pre-immune serum did not cross-react with any of the proteins. On immunoblots of the gels of the purified preparation of soluble starch synthase from mature Estima tubers, the antiserum recognised strongly the two proteins to which it was raised. The antiserum also recognised a minor protein of 140 kDa. On immunoblots of gels of partially purified soluble starch synthase from mature Estima tubers, the antiserum recognised proteins of 140 kDa and 120 kDa. On immunoblots of gels of starch-granule-bound proteins from mature Estima tubers, the antiserum recognised a protein of 140 kDa. There were some faint indications that a 120 kDa protein on the starch was also recognised. A protein of 140 kDa was recognised by the antiserum both in the soluble extracts and on starch granules of developing tubers of Desiree. The 120 kDa protein was very weakly detectable in soluble extracts from these tubers, which also contained a lower molecular weight protein (approximately 100 kDa) recognised

synthase from potato precipitates 75 % of the total soluble starch synthase activity in crude extract (Figure 5). The remainder of the activity is partly due to GBSS II (Table 2), but the possibility of further minor isoforms cannot be ruled out.

The purified soluble starch synthase is likely to represent a novel class of starch synthase. It is not related to the major soluble starch synthase in pea embryo (GBSS II), which is clearly related to the minor, soluble 92 kDa GBSS II in potato. The soluble starch synthase is only very weakly recognised by the antibody raised to GBSS II from pea. It is not related to the GBSS I proteins either: the starch synthase from potato tuber is not recognised by the antibody raised to GBSS I from pea embryo. These results reinforce the view that storage organs differ profoundly in the nature and number of active isoforms of starch synthase (Smith *et al.*, 1995 Plant Physiol. *107*, 1; Edwards *et al.*; and Denyer *et al.*, both cited previously).

Example 2

ISOLATION OF A cDNA CLONE FOR SOLUBLE STARCH SYNTHASE FROM POTATO TUBERS

The antiserum raised to the purified starch synthase protein from Estima tubers was used for immunoscreening of a λgt 11 library (provided by C Grierson, John Innes Centre, Norwich) containing cDNA inserts with *Eco*RI linkers, constructed from developing Estima tuber poly[A] RNA.

Approximately 1.5 x 10^6 plaque-forming units were probed with the antiserum at a dilution of 1/1000. The second antibody was an anti-rat immunoglobulin linked to horseradish peroxidase (Amersham International, Amersham, UK). Two positive clones were isolated. These were both 1.1 kb in length and contained poly(A) tracts at their 3' ends. One of these was cloned into the EcoRI site of pBluescript SK + to give plasmid pRAT2. A 5' *Eco*RI-*ECORV* fragment from this clone was used as a probe on the λgt11 library. Filters were washed in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5 g L^{-1} SDS at 65°C. Seven clones of 1.3, 1.53, 1.75, 1.88, 2.15, 2.21, and 2.4 kb were isolated. The longest clone was subcloned as an *Eco*RI fragment into pBluescript SK+ to give plasmid pRAT20. A 600-bp 5' fragment from pRAT20 was used to probe a random primed λgt11 library prepared from cDNA of developing tubers. Three positive clones were isolated. The longest was 2.3 kb and was subcloned as an *Eco*RI fragment into pBluescript SK+ to give pRAT24.

The 2.3 and 2.4kb partial clones overlapped. The full-length composite cDNA was 4.127kb. The DNA sequence of the full length cDNA, and the predicted polypeptide sequence, are shown in Figure 6. DNA sequences were determined according to Sanger *et al.* (1977) by using Sequenase™ (United States Biochemical). Sequence data were analysed using the Genetics Computer Group (Madison, WI) computer program (Devereux *et al.* 1984 Nucl. Acids Res. 12, 387-395).

To check the identity of the cDNA, the amino acid sequence it predicted was compared with amino acid sequences of two peptides obtained by digestion with endoproteinase Lys-C of the 110-kD protein purified from tubers of cultivar Estima. The peptide sequences FIPIPYTSENVVEGK (Seq. ID No. 1) and HIPVFGG (Seq. ID No. 2) corresponded precisely to predicted sequences from the clone. Attempts to obtain N-terminal amino acid sequence of the purified proteins for comparison with the sequence predicted from the cDNA clone were unsuccessful.

On RNA gel blots of poly(A)$^+$RNA from developing tubers, a partial cDNA clone recognised a single transcript of ~4 kb. This size is considerably greater than those of the transcripts for GBSSI and GBSSII and is consistent with the transcript encoding a protein in the range of 110 to 140 kD.

The deduced amino acid sequence of the soluble starch synthase revealed a protein of 1230 amino acids and a predicted size of 139 kD (Figure 6). At the N terminus was a sequence of ~60 amino acids rich in serine and basic residues and low in acidic residues, which is typical of a chloroplast transit peptide. Based on the consensus of Gavel and von Heinje (1990 FEBS Lett. 261, 455-458), the most likely cleavage site would be between amino acids 60 (Cys) and 61 (Ala), because the serine-rich region ends before this point. Cleavage in this region would give a mature protein of ~132 kD. The structure is somewhat similar to that of GBSSII in that it contains a C-terminal region homologous with starch synthases and bacterial glycogen synthases and an N-terminal extension. The N-terminal extension shows little sequence similarity to the N-terminal extensions of GBSSII from pea or potato (in turn, they show little similarity to each other; Edwards *et al.*, (1995) Plant J. 8, 283-294) or to any other sequence in the data bases. The N-terminal domain resembles those of pea and potato GBSSII in that it shows considerable predicted flexibility (Chou-Fasman algorithm; see Dry *et al.*, (1992) Plant J. 2, 193-202); all these extensions may therefore serve similar roles. At the C-terminal end of the N-terminal extension of the soluble starch synthase are two proline residues; multiple proline residues have been noted previously at the C-terminal ends of N-terminal extensions of both starch synthases and starch-branching enzymes (Dry *et al.*, (1992) Plant J. 2, 193-202: Burton *et al.*, 1995).

The roles of these N-terminal extensions are not known. but it seems likely that they are involved in determining properties such as interaction with starch polymers rather than contributing to basic catalytic properties. The C-terminal region from amino acid 780 to the end shows greatest similarity to glycogen synthases from bacteria, although there

Table 3. Effects of Reduced Activity of SSS on Soluble and Granule-Bound Starch Synthase Activity and Amylose Content of Starch.

Plant[a]	Soluble Activity[b] (nmol $min^{-1}g^{-1}$ Fresh Weight)	Granule-Bound Activity[c] (nmol $min^{-1}g^{-1}$ Fresh Weight)	Amylose Content[d] (% Total Starch)
1	ND[e]	ND	27.8
2	ND	ND	29.5, 29.8
9	18.3 ± 3.9 (4)	118	28.6
18	23.6 ± 6.7 (3)	97	29.3
25	29.5 ± 3.6 (4)	113	27.3
26	33.3 ± 8.3 (3)	80	30.1
Control	98.4 ± 4.9 (9)	106 ± 12	26.4, 28.9
Desiree	ND	ND	27.8, 29.2

[a] Plant numbers refer to individual transgenic plants with reduced SSS activity. Tubers are from a single plant, except for the control line, in which three different plants (each an independent, control transformant) were used.

[b] Soluble activity was measured by using duplicate samples from tubers of 12 to 70 g fresh weight harvested at intervals during plant development. Values are the means ± SE of measurements made with the number of tubers given within parentheses.

[c] Granule-bound activities are the means of measurements made by using duplicate samples from a single tuber (12 to 70 g fresh weight) harvested at maturity.

[d] Amylose content was measured by using starch extracted from two or three tubers per mature plant. Values are the means of measurements made with two separate samples taken from the bulk starch preparations: two values are given when independent starch preparations were used. Wild-type Desiree plants used for these measurements were grown in the same greenhouse at the same time as the transgenic

weight in line 9 and 8.8 nmol $min^{-1}g^{-1}$ fresh weight in control tubers. This indicates that the reduction in SSS has little effect on the soluble activity of GBSSII.

Example 4

Detailed analysis of starch from tubers obtained from transformed Potato plants

Despite the results of crude analysis described in Example 3, indicating that the starch from transformed plants was essentially unaltered, it was decided to perform more detailed analysis of the starch, by Differential Scanning Calorimetry and Viscoamylograph. Analysis was performed as described in WO 95/26407 and WO 96/34968.

Surprisingly it was found that certain physical properties of the starch were consistently significantly altered. In particular, it was found that the viscosity onset temperature was significantly reduced compared to starch obtained from equivalent control plants which did not contain the SSS antisense construct. The results are shown in Table 4.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: National Starch and Chemical Investment Holding Corporation
(B) STREET: Suite 27. 501 Silverside Road
(C) CITY: Wilmington
(D) STATE: Delaware
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): DE 19809

(ii) TITLE OF INVENTION: Improvements in or Relating to Soluble Starch Synthase

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0. Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Phe	Ile	Pro	Ile	Pro	Tyr	Thr	Ser	Glu	Asn	Val	Val	Glu	Gly	Lys
1				5					10					15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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CCT ATT CTT GGG TTT GTC TCT CAT GGA ACC ACA AGT CTA TCA GTA CAA   268
Pro Ile Leu Gly Phe Val Ser His Gly Thr Thr Ser Leu Ser Val Gln
                -30                 -25                 -20

TCT TCT TCA TGG AGG AAG GAT GGA ATG GTT ACT GGG GTT TCA TTT TCC   316
Ser Ser Ser Trp Arg Lys Asp Gly Met Val Thr Gly Val Ser Phe Ser
            -15                 -10                  -5

ATT TGT GCA AAT TTC TCG GGA AGA AGA CGG AGA AAA GTT TCA ACT CCT   364
Ile Cys Ala Asn Phe Ser Gly Arg Arg Arg Arg Lys Val Ser Thr Pro
        1               5                  10

AGG AGT CAA GGC TCT TCA CCT AAG GGG TTT GTG CCA AGG AAG CCC TCA   412
Arg Ser Gln Gly Ser Ser Pro Lys Gly Phe Val Pro Arg Lys Pro Ser
    15                  20                  25                  30

GGG ATG AGC ACG CAA AGA AAG GTT CAG AAG AGC AAT GGT GAT AAA GAA   460
Gly Met Ser Thr Gln Arg Lys Val Gln Lys Ser Asn Gly Asp Lys Glu
                35                  40                  45

AGT AAA AGT ACT TCA ACA TCT AAA GAA TCT GAA ATT TCC AAC CAG AAG   508
Ser Lys Ser Thr Ser Thr Ser Lys Glu Ser Glu Ile Ser Asn Gln Lys
            50                  55                  60

ACG GTT GAA GCA AGA GTT GAA ACT AGT GAC GAT GAC ACT AAA GGA GTG   556
Thr Val Glu Ala Arg Val Glu Thr Ser Asp Asp Asp Thr Lys Gly Val
        65                  70                  75

GTG AGG GAC CAC AAG TTT CTG GAG GAT GAG GAT GAA ATC AAT GGT TCT   604
Val Arg Asp His Lys Phe Leu Glu Asp Glu Asp Glu Ile Asn Gly Ser
    80                  85                  90

ACT AAA TCA ATA AGT ATG TCA CCT GTT CGT GTA TCA TCT CAA TTT GTT   652
Thr Lys Ser Ile Ser Met Ser Pro Val Arg Val Ser Ser Gln Phe Val
95                  100                 105                 110

GAA AGT GAA GAA ACT GGT GGT GAT GAC AAG GAT GCT GTA AAG TTA AAC   700
Glu Ser Glu Glu Thr Gly Gly Asp Asp Lys Asp Ala Val Lys Leu Asn
                115                 120                 125

AAA TCA AAG AGA TCG GAA GAG AGT GGT TTT ATA ATT GAT TCT GTA ATA   748
Lys Ser Lys Arg Ser Glu Glu Ser Gly Phe Ile Ile Asp Ser Val Ile
            130                 135                 140

AGA GAA CAA AGT GGA TCT CAG GGG GAA ACT AAT GCC AGT AGC AAG GGA   796
Arg Glu Gln Ser Gly Ser Gln Gly Glu Thr Asn Ala Ser Ser Lys Gly
        145                 150                 155

AGC CAT GCT GTG GGT ACA AAA CTT TAT GAG ATA TTG CAG GTG GAT GTT   844
Ser His Ala Val Gly Thr Lys Leu Tyr Glu Ile Leu Gln Val Asp Val
    160                 165                 170

GAG CCA CAA CAA TTG AAA GAA AAT AAT GCT GGG AAT GTT GAA TAC AAA   892
Glu Pro Gln Gln Leu Lys Glu Asn Asn Ala Gly Asn Val Glu Tyr Lys
175                 180                 185                 190
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GCT GAA ATT GAA GCT GAC AGA GCA CAA GCA AAG GAA GAG GCT GCA AAG  1612
Ala Glu Ile Glu Ala Asp Arg Ala Gln Ala Lys Glu Glu Ala Ala Lys
415                 420                 425                 430

AAA AAG AAA GTA TTG CGA GAA TTG ATG GTA AAA GCC ACG AAG ACT CGT  1660
Lys Lys Lys Val Leu Arg Glu Leu Met Val Lys Ala Thr Lys Thr Arg
                435                 440                 445

GAT ATC ACC TGG TAC ATA GAG CCA AGT GAA TTT AAA TGC GAG GAC AAG  1708
Asp Ile Thr Trp Tyr Ile Glu Pro Ser Glu Phe Lys Cys Glu Asp Lys
            450                 455                 460

GTC AGG TTA TAC TAT AAC AAA AGT TCA GGT CCT CTC TCC CAT GCT AAG  1756
Val Arg Leu Tyr Tyr Asn Lys Ser Ser Gly Pro Leu Ser His Ala Lys
        465                 470                 475

GAC TTG TGG ATC CAC GGA GGA TAT AAT AAT TGG AAG GAT GGT TTG TCT  1804
Asp Leu Trp Ile His Gly Gly Tyr Asn Asn Trp Lys Asp Gly Leu Ser
    480                 485                 490

ATT GTC AAA AAG CTT GTT AAA TCT GAG AGA ATA GAT GGT GAT TGG TGG  1852
Ile Val Lys Lys Leu Val Lys Ser Glu Arg Ile Asp Gly Asp Trp Trp
495                 500                 505                 510

TAT ACA GAG GTT GTT ATT CCT GAT CAG GCA CTT TTC TTG GAT TGG GTT  1900
Tyr Thr Glu Val Val Ile Pro Asp Gln Ala Leu Phe Leu Asp Trp Val
                515                 520                 525

TTT GCT GAT GGT CCA CCC AAG CAT GCC ATT GCT TAT GAT AAC AAT CAC  1948
Phe Ala Asp Gly Pro Pro Lys His Ala Ile Ala Tyr Asp Asn Asn His
            530                 535                 540

CGC CAA GAC TTC CAT GCC ATT GTC CCC AAC CAC ATT CCG GAG GAA TTA  1996
Arg Gln Asp Phe His Ala Ile Val Pro Asn His Ile Pro Glu Glu Leu
        545                 550                 555

TAT TGG GTT GAG GAA GAA CAT CAG ATC TTT AAG ACA CTT CAG GAG GAG  2044
Tyr Trp Val Glu Glu Glu His Gln Ile Phe Lys Thr Leu Gln Glu Glu
    560                 565                 570

AGA AGG CTT AGA GAA GCG GCT ATG CGT GCT AAG GTT GAA AAA ACA GCA  2092
Arg Arg Leu Arg Glu Ala Ala Met Arg Ala Lys Val Glu Lys Thr Ala
575                 580                 585                 590

CTT CTG AAA ACT GAA ACA AAG GAA AGA ACT ATG AAA TCA TTT TTA CTG  2140
Leu Leu Lys Thr Glu Thr Lys Glu Arg Thr Met Lys Ser Phe Leu Leu
                595                 600                 605

TCT CAG AAG CAT GTA GTA TAT ACT GAA CCT CTT GAT ATC CAA GCT GGA  2188
Ser Gln Lys His Val Val Tyr Thr Glu Pro Leu Asp Ile Gln Ala Gly
            610                 615                 620

AGC AGC GTC ACA GTT TAC TAT AAT CCC GCC AAT ACA GTA CTT AAT GGT  2236
Ser Ser Val Thr Val Tyr Tyr Asn Pro Ala Asn Thr Val Leu Asn Gly
        625                 630                 635
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TAT ACA CAC TAT GGT CTA AGC AAA TCT CGT ATA GTC TTC ACG ATA CAT  2956
Tyr Thr His Tyr Gly Leu Ser Lys Ser Arg Ile Val Phe Thr Ile His
        865                 870                 875

AAT CTT GAA TTT GGG GCA GAT CTC ATT GGG AGA GCA ATG ACT AAC GCA  3004
Asn Leu Glu Phe Gly Ala Asp Leu Ile Gly Arg Ala Met Thr Asn Ala
    880                 885                 890

GAC AAA GCT ACA ACA GTT TCA CCA ACT TAC TCA CAG GAG GTG TCT GGA  3052
Asp Lys Ala Thr Thr Val Ser Pro Thr Tyr Ser Gln Glu Val Ser Gly
895                 900                 905                 910

AAC CCT GTA ATT GCG CCT CAC CTT CAC AAG TTC CAT GGT ATA GTG AAT  3100
Asn Pro Val Ile Ala Pro His Leu His Lys Phe His Gly Ile Val Asn
                915                 920                 925

GGG ATT GAC CCA GAT ATT TGG GAT CCT TTA AAC GAT AAG TTC ATT CCG  3148
Gly Ile Asp Pro Asp Ile Trp Asp Pro Leu Asn Asp Lys Phe Ile Pro
            930                 935                 940

ATT CCG TAC ACT TCA GAA AAC GTT GTT GAG GGC AAA ACA GCA GCC AAG  3196
Ile Pro Tyr Thr Ser Glu Asn Val Val Glu Gly Lys Thr Ala Ala Lys
        945                 950                 955

GAA GCT TTG CAG CGA AAA CTT GGA CTG AAA CAG GCT GAC CTT CCT TTG  3244
Glu Ala Leu Gln Arg Lys Leu Gly Leu Lys Gln Ala Asp Leu Pro Leu
    960                 965                 970

GTA GGA ATT ATC ACC CGC TTA ACT CAC CAG AAA GGA ATC CAC CTC ATT  3292
Val Gly Ile Ile Thr Arg Leu Thr His Gln Lys Gly Ile His Leu Ile
975                 980                 985                 990

AAA CAT GCT ATT TGG CGC ACC TTG GAA CGG AAC GGA CAG GTA GTC TTG  3340
Lys His Ala Ile Trp Arg Thr Leu Glu Arg Asn Gly Gln Val Val Leu
                995                 1000                1005

CTT GGT TCT GCT CCT GAT CCT AGG GTA CAA AAC AAT TTT GTT AAT TTG  3388
Leu Gly Ser Ala Pro Asp Pro Arg Val Gln Asn Asn Phe Val Asn Leu
            1010                1015                1020

GCA AAT CAA TTG CAC TCC AAA TAT AAT GAC CGC GCA CGA CTC TGT CTA  3436
Ala Asn Gln Leu His Ser Lys Tyr Asn Asp Arg Ala Arg Leu Cys Leu
        1025                1030                1035

ACA TAT GAC GAG CCA CTT TCT CAC CTG ATA TAT GCT GGT GCT GAT TTT  3484
Thr Tyr Asp Glu Pro Leu Ser His Leu Ile Tyr Ala Gly Ala Asp Phe
    1040                1045                1050

ATT CTA GTT CCT TCA ATA TTT GAG CCA TGT GGA CTA ACA CAA CTT ACC  3532
Ile Leu Val Pro Ser Ile Phe Glu Pro Cys Gly Leu Thr Gln Leu Thr
1055                1060                1065                1070

GCT ATG AGA TAT GGT TCA ATT CCA GTC GTG CGT AAA ACT GGA GGA CTT  3580
Ala Met Arg Tyr Gly Ser Ile Pro Val Val Arg Lys Thr Gly Gly Leu
                1075                1080                1085
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Gly Arg Arg Arg Arg Lys Val Ser Thr Pro Arg Ser Gln Gly Ser Ser
5 10 15 20

Pro Lys Gly Phe Val Pro Arg Lys Pro Ser Gly Met Ser Thr Gln Arg
25 30 35

Lys Val Gln Lys Ser Asn Gly Asp Lys Glu Ser Lys Ser Thr Ser Thr
40 45 50

Ser Lys Glu Ser Glu Ile Ser Asn Gln Lys Thr Val Glu Ala Arg Val
55 60 65

Glu Thr Ser Asp Asp Asp Thr Lys Gly Val Val Arg Asp His Lys Phe
70 75 80

Leu Glu Asp Glu Asp Glu Ile Asn Gly Ser Thr Lys Ser Ile Ser Met
85 90 95 100

Ser Pro Val Arg Val Ser Ser Gln Phe Val Glu Ser Glu Glu Thr Gly
105 110 115

Gly Asp Asp Lys Asp Ala Val Lys Leu Asn Lys Ser Lys Arg Ser Glu
120 125 130

Glu Ser Gly Phe Ile Ile Asp Ser Val Ile Arg Glu Gln Ser Gly Ser
135 140 145

Gln Gly Glu Thr Asn Ala Ser Ser Lys Gly Ser His Ala Val Gly Thr
150 155 160

Lys Leu Tyr Glu Ile Leu Gln Val Asp Val Glu Pro Gln Gln Leu Lys
165 170 175 180

Glu Asn Asn Ala Gly Asn Val Glu Tyr Lys Gly Pro Val Ala Ser Lys
185 190 195

Leu Leu Glu Ile Thr Lys Ala Ser Asp Val Glu His Thr Glu Ser Asn
200 205 210

Glu Ile Asp Asp Leu Asp Thr Asn Ser Phe Phe Lys Ser Asp Leu Ile
215 220 225

Glu Glu Asp Glu Pro Leu Ala Ala Gly Thr Val Glu Thr Gly Asp Ser
230 235 240

Ser Leu Asn Leu Arg Leu Glu Met Glu Ala Asn Leu Arg Arg Gln Ala
245 250 255 260

Ile Glu Arg Leu Ala Glu Glu Asn Leu Leu Gln Gly Ile Arg Leu Phe
265 270 275

Cys Phe Pro Glu Val Val Lys Pro Asp Glu Asp Val Glu Ile Phe Leu
280 285 290

Asn Arg Gly Leu Ser Thr Leu Lys Asn Glu Ser Asp Val Leu Ile Met
295 300 305

Tyr Thr Glu Pro Leu Asp Ile Gln Ala Gly Ser Ser Val Thr Val Tyr
615 620 625

Tyr Asn Pro Ala Asn Thr Val Leu Asn Gly Lys Pro Glu Ile Trp Phe
630 635 640

Arg Cys Ser Phe Asn Arg Trp Thr His Arg Leu Gly Pro Leu Pro Pro
645 650 655 660

Gln Lys Met Ser Pro Ala Glu Asn Gly Thr His Val Arg Ala Thr Val
665 670 675

Lys Val Pro Leu Asp Ala Tyr Met Met Asp Phe Val Phe Ser Glu Arg
680 685 690

Glu Asp Gly Gly Ile Phe Asp Asn Lys Ser Gly Met Asp Tyr His Ile
695 700 705

Pro Val Phe Gly Gly Val Ala Lys Glu Pro Pro Met His Ile Val His
710 715 720

Ile Ala Val Glu Met Ala Pro Ile Ala Lys Val Gly Gly Leu Gly Asp
725 730 735 740

Val Val Thr Ser Leu Ser Arg Ala Val Gln Asp Leu Asn His Asn Val
745 750 755

Asp Ile Ile Leu Pro Lys Tyr Asp Cys Leu Lys Met Asn Asn Val Lys
760 765 770

Asp Phe Arg Phe His Lys Asn Tyr Phe Trp Gly Gly Thr Glu Ile Lys
775 780 785

Val Trp Phe Gly Lys Val Glu Gly Leu Ser Val Tyr Phe Leu Glu Pro
790 795 800

Gln Asn Gly Leu Phe Ser Lys Gly Cys Val Tyr Gly Cys Ser Asn Asp
805 810 815 820

Gly Glu Arg Phe Gly Phe Phe Cys His Ala Ala Leu Glu Phe Leu Leu
825 830 835

Gln Gly Gly Phe Ser Pro Asp Ile Ile His Cys His Asp Trp Ser Ser
840 845 850

Ala Pro Val Ala Trp Leu Phe Lys Glu Gln Tyr Thr His Tyr Gly Leu
855 860 865

Ser Lys Ser Arg Ile Val Phe Thr Ile His Asn Leu Glu Phe Gly Ala
870 875 880

Asp Leu Ile Gly Arg Ala Met Thr Asn Ala Asp Lys Ala Thr Thr Val
885 890 895 900

Ser Pro Thr Tyr Ser Gln Glu Val Ser Gly Asn Pro Val Ile Ala Pro
905 910 915

compared to starch extracted from equivalent, non-transformed plants.

2. Altered starch according to claim 1, wherein the viscosity onset temperature is reduced by at least 7°C compared to starch extracted from equivalent, non-transformed plants.

3. Altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry, of less than 60°C.

4. Altered starch according to claim 3 which, as extracted, has a viscosity onset temperature of less than 55°C.

5. Altered starch according to any one of claims 1-4 which, as extracted, has a reduced endotherm peak temperature (as extracted) as determined by differential scanning calorimetry compared to starch extracted from equivalent, non-transformed plants.

6. Altered starch according to any one of claims 1-5 which, as extracted, has an endotherm peak temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants.

7. Altered starch according to any one of claims 1-6 which, as extracted, has an endotherm peak temperature as determined by differential scanning calorimetry, of less than 59°C.

8. Altered starch according to any one of the preceding claims, having a substantially normal amylose content.

9. A polypeptide obtainable from a soluble extract of potato tubers and having starch synthase activity, in substantially isolated form.

10. A polypeptide according to claim 9, having an apparent molecular weight, as judged by SDS-PAGE, in the range of 100-140 kDa, or a functional equivalent thereof.

11. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 140 kDa.

12. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 120 kDa.

13. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 110 kDa.

14. A polypeptide according to claim 9 or 10, obtainable from developing tubers of S. *tuberosum* cultivar Desirée, having an apparent molecular weight, as judged by SDS-PAGE, of 100 kDa.

15. A polypeptide according to any one of claims 9-14, comprising the amino acid sequence shown in Figure 6.

16. A nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or antisense orientation to a promoter operable in a plant.

17. A sequence according to claim 16 comprising at least 300-600bp.

18. A sequence according to claim 16 or 17, exhibiting at least 85% sequence identity with the corresponding region of the DNA sequence shown in Figure 6.

19. A sequence according to any one of claims 16, 17 or 18 exhibiting at least 90% sequence identity with the corresponding region of the DNA sequence shown in Figure 6.

20. A sequence according to any one of claims 16-19, comprising a 5' and/or a 3' untranslated region.

21. A sequence according to any one of claims 16-20, encoding at least a portion of a polypeptide in accordance with

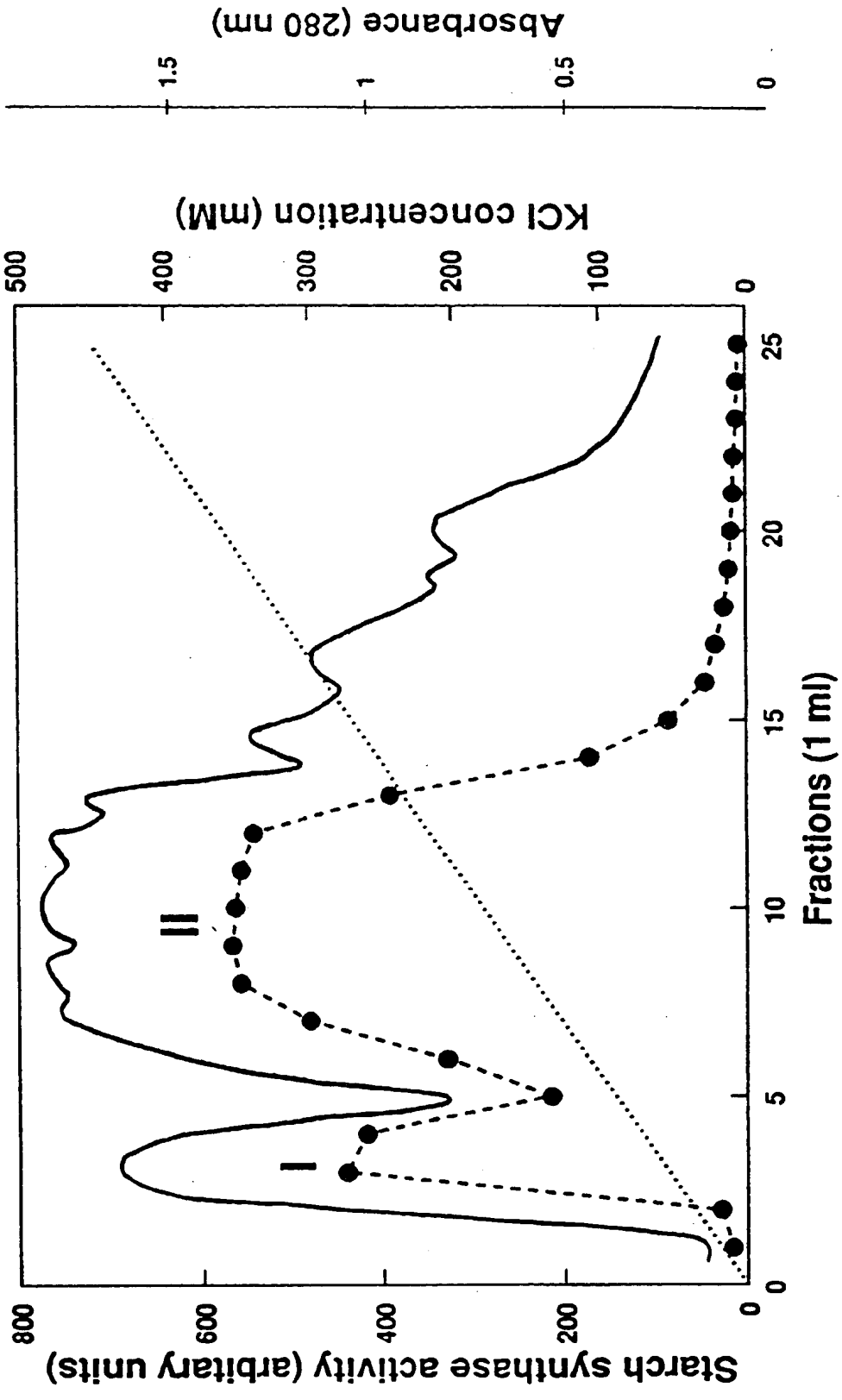


Fig. 1

Fig. 3

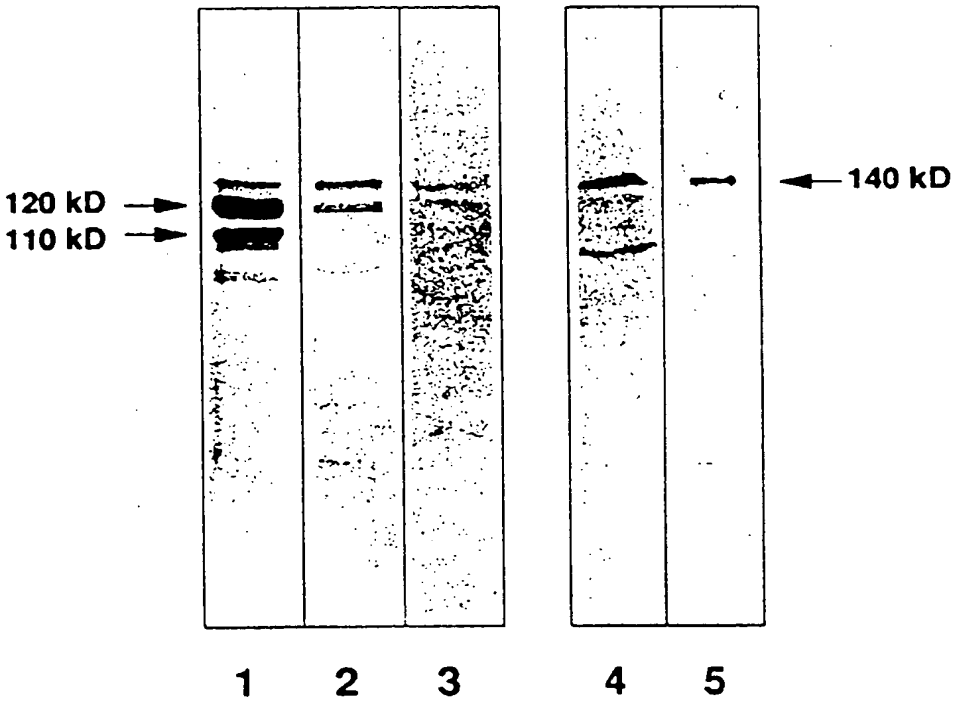


Fig. 5

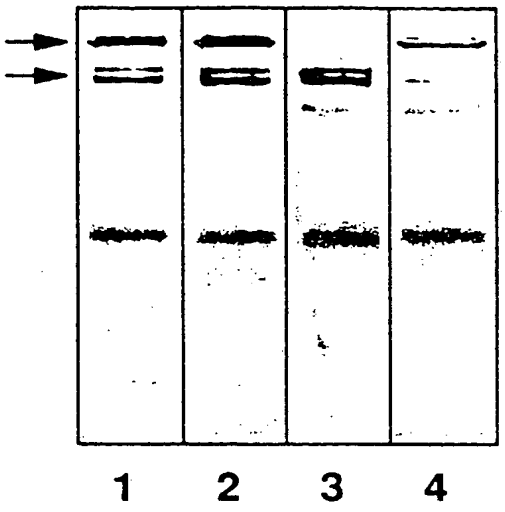


Fig. 6 (con't)

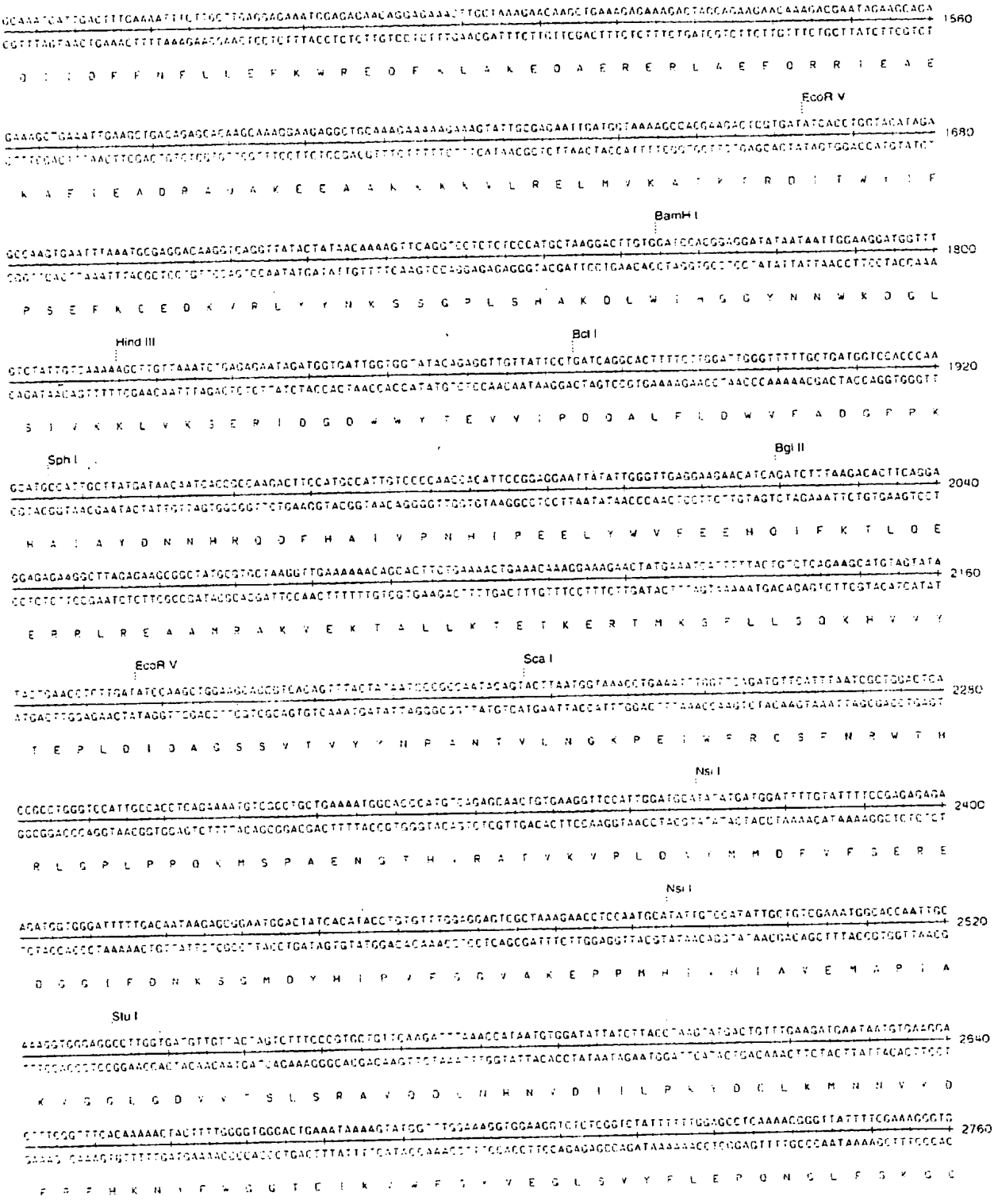
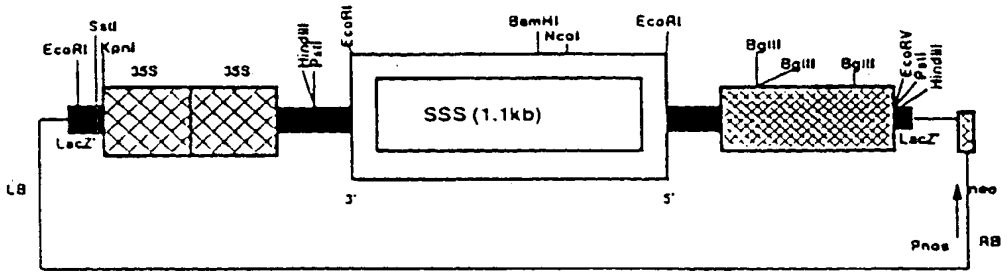


Fig. 7

A

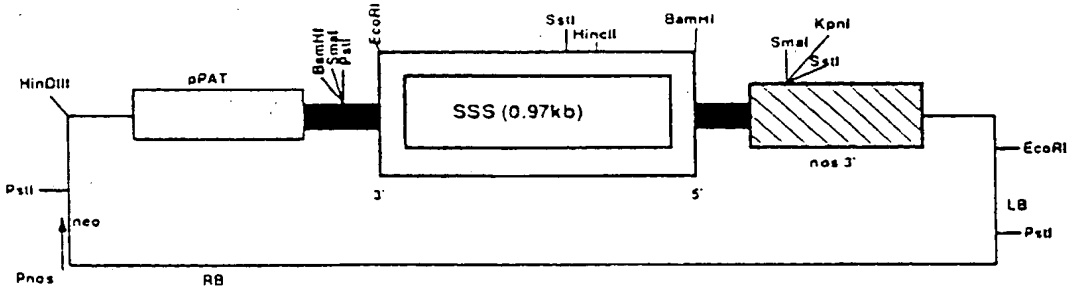
pRAT4



BIN19

B

pPATRAT



pBI140.5

2x 35S promoter

CaMV polyA

Polylinker

LB, RB Left and right border DNA

Patatin promoter

Nopaline synthase terminator

Neo Neomycin phosphotransferase

Pnos Nopaline synthase promoter

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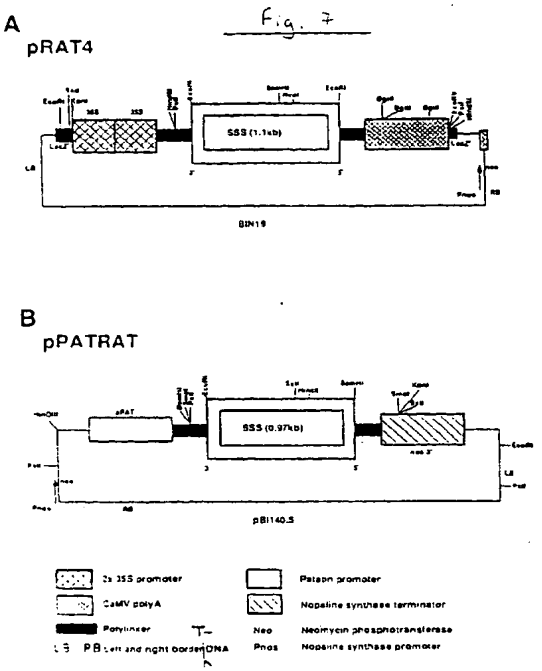
(54) Improvements in or relating to soluble starch synthase

(57) Disclosed is altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants, together with a nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or antisense orientation to a promoter operable in a plant, and host cells and plants comprising the sequence.

Fig. 7



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